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Identification of a BET Family Bromodomain/Casein Kinase II/TAF-Containing Complex as a Regulator of Mitotic Condensin Function

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SUMMARY

Condensin is a central regulator of mitotic genome structure with mutants showing poorly condensed chromosomes and profound segregation defects. Here, we identify NCT, a complex comprising the *N*rc1 BET-family tandem bromodomain protein (SPAC631.02), *c*asein kinase II (CKII), and several *T*AFs, as a regulator of condensin function. We show that NCT and condensin bind similar genomic regions but only briefly colocalize during the periods of chromosome condensation and decondensation. This pattern of NCT binding at the core centromere, the region of maximal condensin enrichment, tracks the abundance of acetylated histone H4, as regulated by the Hat1-Mis16 acetyltransferase complex and recognized by the first Nrc1 bromodomain. Strikingly, mutants in NCT or Hat1-Mis16 restore the formation of segregation-competent chromosomes in cells containing defective condensin. These results are consistent with a model where NCT targets CKII to chromatin in a cell-cycle-directed manner in order to modulate the activity of condensin during chromosome condensation and decondensation.

INTRODUCTION

The genome has to be faithfully transmitted to each daughter at cell division. To this end, the interphase chromatin is condensed to individual chromosomes at early mitosis, providing the structure needed to survive sister-chromatid separation. A major regulator of this extensive remodeling is the pentameric condensin complex, comprising two SMC (structural maintenance of chromosomes) ATPases, a kleisin, and two HEAT-repeat proteins (Cuylen and Haering, 2011; Hirano, 2012; Onn et al., 2007; Wood et al., 2010). Cells containing mutants in each condensin subunit show poorly structured mitotic chromosomes and profound segregation defects, including the fission yeast (*Schizosaccharomyces pombe*; *Sp*) *cut*$^{+}$ phenotype where the division septum cuts through unsegregated chromosomes at the metaphase plate (Saka et al., 1994).

Many metazoans contain two condensin complexes (I and II) that pair the same SMCs with alternative accessory subunits. This allows each complex to function independently, such that vertebrate condensin II regulates early chromosome condensation in prophase, and condensin I then loads in prometaphase to complete the reaction (Hirota et al., 2004; Ono et al., 2003, 2004). Fission yeast, in contrast, relies on a single condensin I that is presumed to regulate chromosome condensation through mitosis. The precise means by which any condensin regulates chromosome structure is unclear. In vitro analyses show that the immunopurified complex can introduce positive supercoils to relaxed circular DNA (in concert with

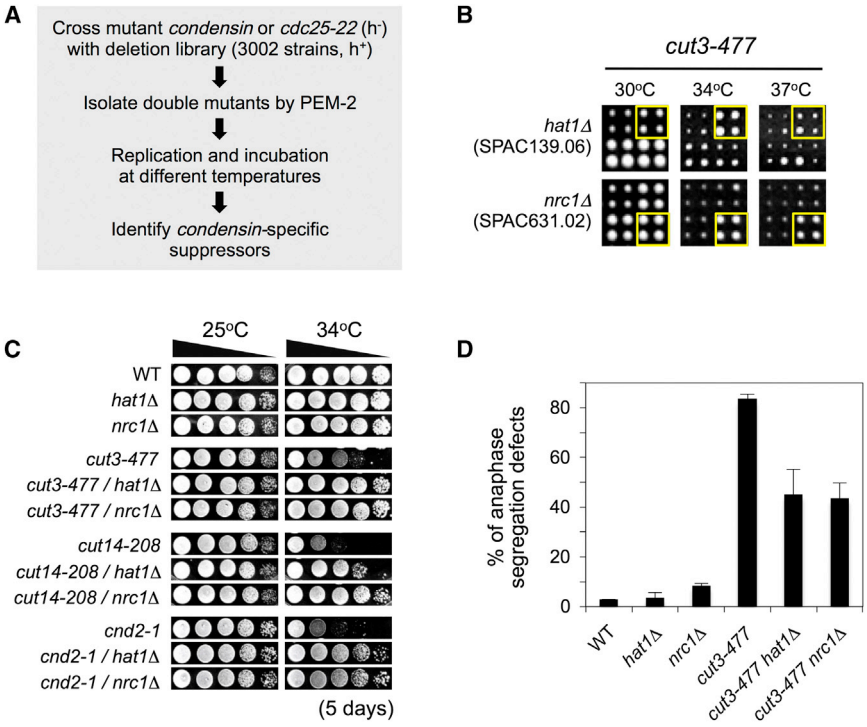

Figure 1. Deletions of the Hat1 Acetyltransferase and Nrc1 Bromodomain Rescue Mutant Condensin

(A) The *PEM-2* approach was used to place conditional temperature-sensitive (*ts*) alleles of condensin (*cut3-477*, *cut14-208*) or cdc25 phosphatase (*cdc25-22*) in the context of deletions of ~75% of the nonessential *Sp* genome.

(B) *condensin*-specific suppressors include individual deletions of Hat1 acetyltransferase and the Nrc1 bromodomain. Double-mutant arrays were pinned in quadruplicate and incubated as indicated (mutants of interest are boxed; panel are size standardized to facilitate cross-comparison).

(C) *hat1*Δ and *nrc1*Δ rescue condensin *ts* alleles (*cut3-477*, *cut14-208*, and *cnd2-1*) in direct testing. Strains were isolated by crossing and tetrad dissection, spotted as 10-fold serial dilutions onto nonselective YES media, and incubated as indicated. WT, wild-type.

(D) *hat1*Δ and *nrc1*Δ repair the anaphase chromosome segregation defects of *cut3-477*. Replicate cultures were shifted from 25°C to 34°C (nonpermissive for *cut3-477*) for 3 hr and fixed, and the percentage (mean ± SD) of late anaphase cells with chromosome segregation defects was determined.

topoisomerase I) and induce chiral knotting in nicked DNA (with topoisomerase II) (Kimura et al., 1999). During in vivo condensation, condesin is also thought to generate higher order structures by directly linking distant regions of a chromosome fiber (Cuylen and Haering, 2011; Hirano, 2012; Wood et al., 2010).

Condensin is regulated by multiple means at various cell-cycle stages, including differential compartmentalization, chromosomal association, and covalent modification. In this manner, fission yeast condensin localizes to the cytoplasm for much of the cell cycle but is phosphorylated by Cdc2 at early mitosis and transported into the nucleus for loading to the centromere, rDNA, and specific locations along the chromosome arms (Nakazawa et al., 2008; Sutani et al., 1999). This preference for a range of genomic features is likely mediated by binding of the condensin subunits to various chromatin marks (e.g., H4-K20Me1 and the H2A/H2A.Z N-terminal tails; Liu et al., 2010; Tada et al., 2011) and adaptor proteins (e.g., Csm1/Lrs4, Scc2/Scc4, TFIIIB/TFIIIC, Cti1, Cti2, *PARP*, *PP2A*, *AKAP95*, and Pku80; Chen et al., 2004; D'Ambrosio et al., 2008; Heale et al., 2006; Johzuka and Horiuchi, 2009; Steen et al., 2000; Takemoto et al., 2009; Tanaka et al., 2012).

Chromosome condensation is unlikely a simple direct consequence of condensin-DNA binding: the complex also has to be activated. Covalent modification is presumed central to this regulation, with many of the condensin subunits extensively phosphorylated, acetylated, and sumoylated (Bazile et al., 2010; Choudhary et al., 2009; Cuylen and Haering, 2011; Hirano, 2012). Phosphorylation is the most studied, where distinct events can inhibit (if catalyzed by casein kinase II [CKII]) or activate (if catalyzed by various mitotic kinases) condensins' in vitro supercoiling activity (Bazile et al., 2010). In a related fashion the human *hsp90*, *hsp27*, *hsp70*, and *c-fos* genes recruit *PP2A* phosphatase to accelerate their re-expression in the following G1 by dephosphorylating/inactivating any colocalized condensin (Sarge and Park-Sarge, 2009; Xing et al., 2008).

In this study, we identify regulators of fission yeast condensin, and thus mitotic chromosome function. We describe the Hat1-Mis16 acetyltransferase complex, show that this contributes to the acetylation of histones H3 and H4 at the core centromere (the region of peak condensin loading), and demonstrate that these modifications are cell cycle regulated and anticorrelated with condensin binding through mitosis. We also describe the NCT complex, comprising the Nrc1 bromodomain (SPAC631.02), CKII, and several TAF proteins and show that NCT and condensin bind similar genomic regions, but only briefly colocalize during the periods of chromosome condensation and decondensation. Importantly, we find that mutants in Hat1-Mis16 or NCT restore the formation of segregation-competent chromosomes in cells containing defective condensin. Our findings are consistent with a model where NCT targets CKII to chromatin in a cell-cycle-directed manner to modulate condensins' catalytic activity, thus regulating chromosome condensation and decondensation.

RESULTS

Deletions of the Hat1 Acetyltransferase and Nrc1 Bromodomain Rescue the Anaphase Segregation Defects of Mutant Condensin

To begin this study, we used a genetics approach to identify second site deletions that specifically rescue the lethality of conditional *condensin* mutants (the temperature-sensitive [*ts*] alleles *cut3-477* or *cut14-208*), expecting this would uncover regulators of mitotic chromosome function (Figure 1A). Screening 3,002

fission yeast deletions (~75% of the nonessential genome; Kim et al., 2010) identified suppressive deletions of two factors with likely roles in the regulation or recognition of acetyl-lysine (Kac): Hat1 acetyltransferase and *SPAC631.02* (hereafter called Nrc1; negative regulator of condensin 1), a poorly characterized protein containing a BET-family tandem bromodomain (Figures 1B and S1A). Direct testing confirmed that *hat1*Δ and *nrc1*Δ also suppressed a *ts* allele of the condensin kleisin subunit (*cnd2-1*) (Figure 1C). Furthermore each deletion significantly improved the anaphase chromosome structure achievable by *cut3-477* alone (Figure 1D), indicating that their suppression of mutant *condensin* is mediated at mitosis.

Hat1-Mis16 Regulates the Core Centromeric Histone Acetylation that Anticorrelates with Condensin Occupancy through Mitosis

The identification of *hat1*Δ and *nrc1*Δ as *condensin* suppressors suggested that specific acetyl-lysines might play a role in condensin function. To investigate this, we first examined the core centromere, the region of maximal condensin enrichment at mitosis (Nakazawa et al., 2008) (Figures S1B–S1D). Of note, H4ac at this location is dramatically reduced in a metaphase -arrested population when condensin is maximally loaded (Figure 2A). Hat1 contributes to the acetylation of histones H3 and H4 (Benson et al., 2007) (Figure 2B), so we used chromatin immunoprecipitation (ChIP) to monitor these modifications in cell-cycle synchronized populations. In this approach, H3ac and H4ac levels at the core and outer centromere temporally anticorrelated with condensin: i.e., they reduced as Cut3 bound in prometaphase and increased as Cut3 departed in G_1/S (Figures 2C and S1E–S1I). Hat1 contributed to this acetylation profile, because *hat1*Δ constitutively reduced centromeric H3ac and H4ac levels and delayed mitotic progression and condensin binding (Figures 2D and S1I). These findings identified Hat1 acetyltransferase as a potential regulator of mitosis and centromere function.

We next asked how Hat1 might itself be regulated and sought any attendant proteins by sequential purification/liquid chromatography mass spectrometry (LC-MS) from whole-cell extracts (WCEs) containing Hat1.TAP. This identified Mis16 (~37% identity to Hat2 of the budding yeast Hat1-Hat2 complex) as an associated protein, a relationship confirmed by coimmunoprecipitation (Figure 2E). Mis16 was previously identified in complex with Mis18 (Hayashi et al., 2004), but our reciprocal purifications from Mis16.TAP and Mis18.TAP WCEs distinguished two complexes: [Mis16-Mis18-SPBC27B12.02-SPBC776.16] and [Hat1-Mis16] (Table S1). Thus, fission yeast likely contains at least two Mis16 complexes with distinct functions. The previously described [Mis16-Mis18] regulates the centromeric loading of histone variant CenH3 (*Sp* Cnp1) via the Scm3 chaperone (Hayashi et al., 2004; Pidoux et al., 2009; Williams et al., 2009), whereas the newly identified Hat1-Mis16 may regulate histone acetylation and condensin function in a more general fashion. Supporting this proposal Mis18.GFP is exclusively centromeric, whereas Mis16.GFP shows diffuse nuclear fluorescence with centromeric enrichment (Hayashi et al., 2004).

Previous studies indicate that Mis16 dissociates from the centromere in prometaphase and returns at telophase/G_1 (Hayashi et al., 2004), a pattern that would allow it to contribute to the mitotic regulation of H3ac and H4ac levels at this location (e.g., Figure 2C). To examine this further, we subjected a strain containing epitope-tagged Mis16 to synchronous ChIP and observed that Mis16.HA_3 indeed associated at the core centromere in a manner correlated with H3ac (Figure S1J). However, these cells also showed mutant phenotypes reminiscent of *hat1*Δ, including reduced centromeric H3ac levels, delays in mitotic progression (compare Figures 2D and 2F), and the rescue of *condensin* mutants (Figures 2G and S1K). This may suggest that Mis16 mediates the association of Hat1 with chromatin, using its C-terminal WD40 repeats to target the acetyltransferase to specific locations (Ruthenburg et al., 2006; Trievel and Shilatifard, 2009): epitope-tagging at the Mis16 C terminus could impede this function, and thus phenocopy *hat1*Δ.

The First Bromodomain of Nrc1 Binds H4ac

The temporal anticorrelation of H3ac/H4ac with condensin at the centromere (e.g., Figure 2D) raised the possibility that these modifications could influence the condensation/decondensation of this region. Furthermore, we considered that this could be effector mediated, such that the acetylations recruit a negative regulator of condensin.

The primary module with Kac specificity is the ~110 amino acid bromodomain (BD) (Filippakopoulos et al., 2012), so it was of interest that deletion of the tandem-BD protein Nrc1 also suppressed *condensin* mutants (Figures 1B–1D). Indeed, there was a dose-dependent relationship between these factors, with reduced or increased Nrc1 levels respectively suppressing or enhancing the growth defects of mutant condensin (Figures S2A–S2C). Furthermore, genetic analyses of *nrc1*Δ identified negative interactions with mutants related to chromosome segregation, centromere identity, histone deacetylation, and condensin function (Roguev et al., 2008; Ryan et al., 2012) (Figure S2D). Importantly, the latter group included the deletion (*pht1*Δ) or unacetylatable mutation (*pht1-N*Δ, *4KR*, or *4KQ*) of histone variant H2A.Z, all of which show the premature dissociation of condensin from anaphase chromosomes (Kim et al., 2009). Together, this collection of genetic interactions strongly supported a role for Nrc1 in mitotic chromosome function.

Various models of BDs in complex with specific Kac peptides identify a conserved region comprising a left-handed bundle of four α helices ($\alpha_{Z,\ A,\ B,\ C}$) linked by the ZA and BC loops that contribute to substrate specificity (Dhalluin et al., 1999; Jacobson et al., 2000; Owen et al., 2000). We used these structures to mutagenize the region containing an invariant tyrosine in the ZA loop of each Nrc1 BD (BD1*, *nrc1-PDYP*$_{266\text{-}269}$*AAAA*; BD2*, *nrc1-PDYF*$_{428\text{-}431}$*AAAA*; BD1*/BD2*: Figure 3A), which should significantly reduce their affinity for Kac (Dhalluin et al., 1999). Immunoblotting confirmed that each allele was expressed at a comparable level to wild-type (WT, Figure 3B), but *nrc1-BD1*/BD2** showed reduced centromeric recruitment after mitosis (Figure 3C) indicating that its bromodomains contribute to the association of Nrc1 with chromatin. This appears essential to Nrc1 function because each BD mutant also rescued mutant *condensin* (Figure 3D), recapitulating this phenotype of *nrc1*Δ (e.g., Figure 1C).

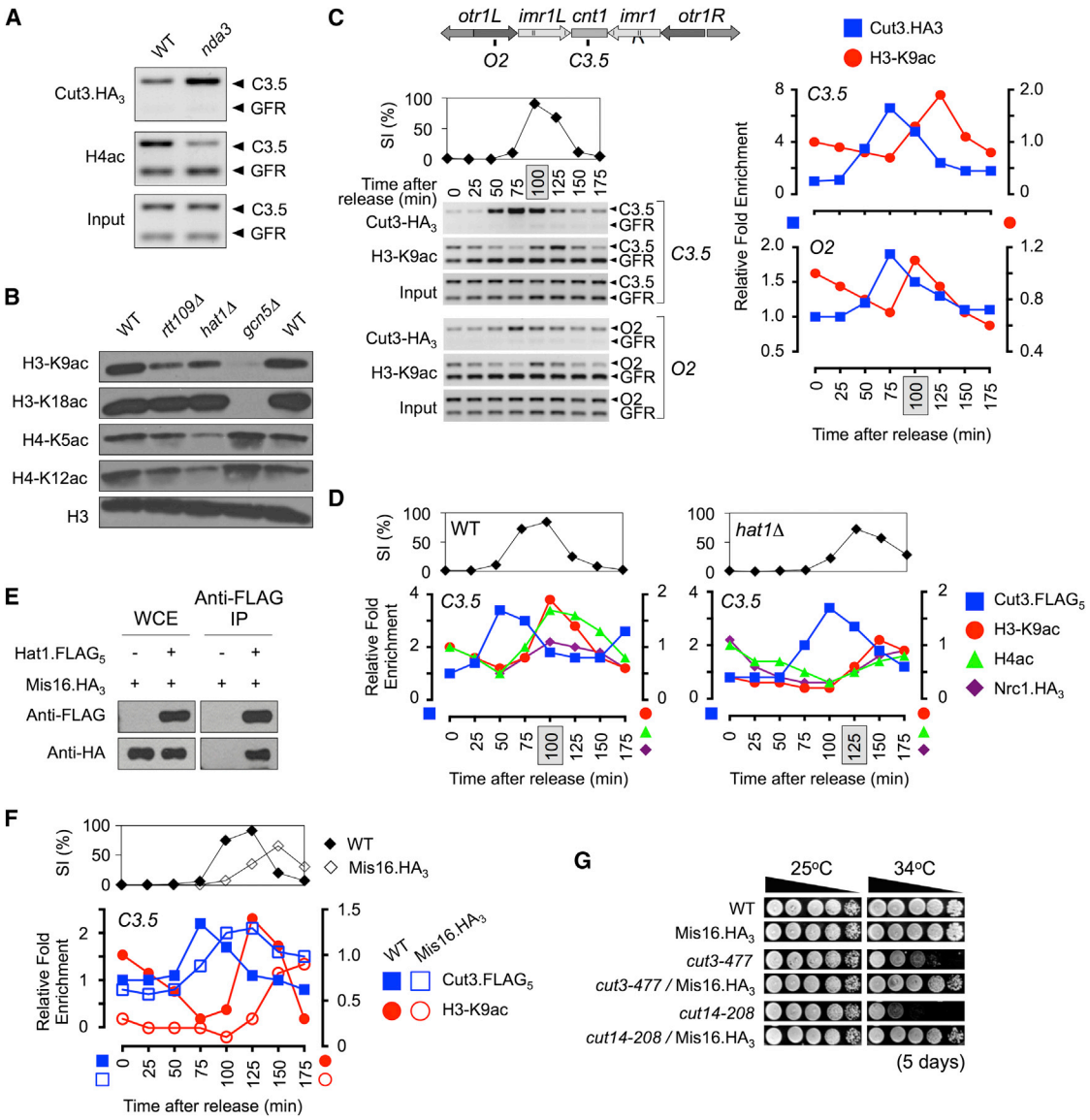

Figure 2. Centromeric H3ac and H4ac Levels Anticorrelate with Condensin through Mitosis in a Hat1-Dependent Manner

(A) H4ac is reduced at the core centromere in metaphase-arrested cells. WT or *nda3-KM311* cells were placed at a restrictive temperature for the cold-sensitive tubulin allele (20°C, 6 hr) to induce spindle-dependent metaphase arrest (Hiraoka et al., 1984), and ChIP was used to monitor condensin (represented by the Cut3 subunit) and H4ac in each population (WT is asynchronous). In each duplex PCR, the upper band is the test region (C3.5, core centromere: see Figure 2C); lower band (GFR, Gene Free Region) is a condensin-free location as a background control (Kim et al., 2009). Input tests primer efficiency in each sample.

(B) Hat1 contributes to the acetylation of histones H3 and H4. Whole-cell extracts (WCEs) were isolated from the indicated strains and the relative level of each acetylated species determined by immunoblotting. Total H3 is a loading control.

(C) Centromeric H3-K9ac levels anticorrelate with condensin. Cells (additionally containing *cdc25-22*) were arrested at G_2/M before synchronous release (as in Figure S1B) and ChIP used to monitor the indicated factors or histone modifications at the chromosome I centromere core or outermost repeats (*C3.5* or *O2*; see upper schematic). The septation index (SI: peak shaded) and control ChIPs confirmed synchronous progression and equivalent antibody access at each time point (see also Figures S1E–S1J). The specific ChIP signal at each location/time point is expressed relative to the respective T0 (set to 1).

(D) Nrc1 binding at the centromere core parallels Hat1-dependent H3-K9ac and H4ac and anticorrelates with condensin (see also Figure S1I). Each ChIP signal is expressed relative to the respective WT T0 (set to 1).

(E) Hat1 coprecipitates Mis16 (see also Table S1). Immunoblots of epitope tagged factors in WCEs or immunoprecipitates (IPs) are as indicated.

(F) Mis16.HA_3 binding at the centromere core parallels mitotically regulated H3-K9ac (see also Figure S1J). The timing of peak septation and condensin binding in Mis16.HA_3 indicates delayed mitotic progression (as in *hat1*Δ: D).

(G) Mis16.HA_3 rescues *condensin* mutants (see also Figure S1K). Strains were spotted as 10-fold serial dilutions onto YES plates.

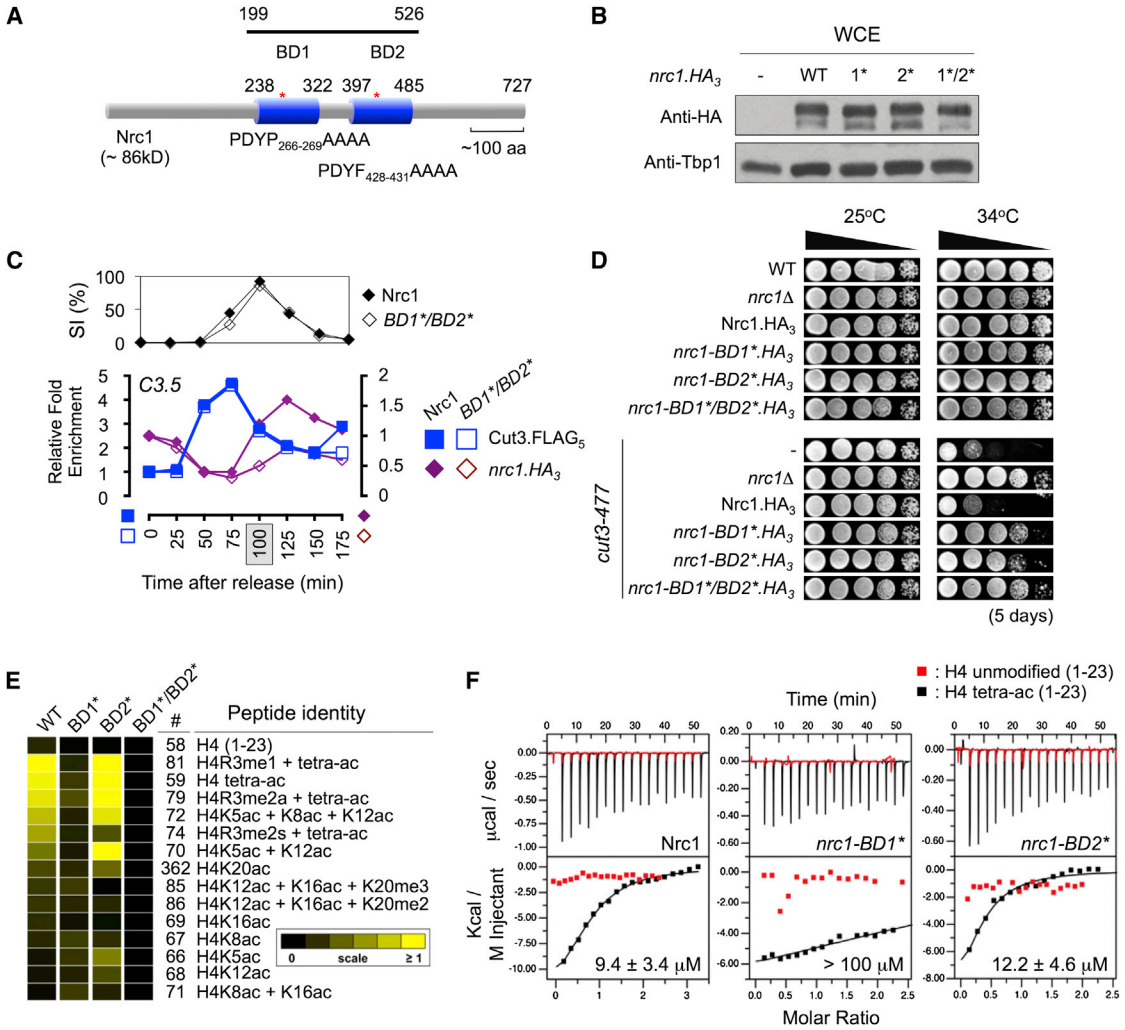

Figure 3. Nrc1-BD1 Specifically Interacts with Hat1-Acetylated Histone H4

(A) Schematic indicates the relative location of both Nrc1 bromodomains, the BD1* and BD2* mutations, and the region cloned for recombinant expression.

(B) Mutation of the αZA loop of each BD has no impact on Nrc1 protein stability. WCEs were isolated, and the relative level of each *nrc1.HA*$_3$ was determined by immunoblotting. Tbp1 is a loading control.

(C) Nrc1 requires its BDs for efficient recruitment to the centromere. Cells were synchronized and ChIPed as in Figure 2C, with the specific ChIP signal at each time point expressed relative to the respective WT T0 (set to 1).

(D) Mutation of the Nrc1 BDs rescues *cut3-477*. Strains were spotted as 10-fold serial dilutions onto YES plates.

(E) Nrc1-BD1 shows strong specificity for the hyperacetylated N terminus of histone H4 (see also Figures S2E–S2G). Purified recombinants were used to probe histone-peptide arrays, and areas of relative enrichment were identified.

(F) Nrc1-BD1 binds with µM affinity to H4tetra-ac but not to unacetylated H4 (both peptides: H4 residues 1–23). Time courses of raw injection heats (isotherm, upper panels) and normalized binding enthalpies are shown.

Individual BDs display selectivity for specific acetyl-lysines, most frequently (though unlikely exclusively) those on the histone N termini (Dhalluin et al., 1999; Filippakopoulos et al., 2012; Owen et al., 2000). To examine such specificity for the Nrc1 BDs, we purified recombinant WT and mutant forms of the domains (Figure 3A) and used each to probe a comprehensive array of histone peptides containing various combinations of posttranslational modifications (Fuchs et al., 2011; Rothbart et al., 2012). In this approach, the WT and *BD2** recombinants (but not *BD1** or *BD1*/BD2**) showed strong specificity for peptides representing a hyperacetylated form of the histone H4 N terminus (Figures 3E and S2E–S2G). This was further investigated by isothermal titration calorimetry (ITC), where the WT and *BD2** recombinants bound with ~10 µM affinity to an H4tetra-ac peptide, an ability not exhibited by *BD1** (Figure 3F). Together, these analyses indicate that Nrc1 binds H4ac via BD1, a specificity that reflects the correlation between centromeric H4ac and Nrc1 levels through mitosis (Figure 2D). Our inability to distinguish Nrc1-BD2 binding in these in vitro studies does not indicate a lack of function, with the importance of this domain demonstrated by phenotypic testing (Figure 3C). It is possible that BD2 binds arrayed histone peptides weaker than the

detection threshold, or recognizes an unrepresented histone or nonhistone Kac substrate.

Identification and Characterization of the NCT Complex

The above results suggest that Nrc1 could be an effector, recruited by specific mitotically regulated histone acetylations to regulate condensin function. However, other than its tandem bromodomains to mediate chromatin binding, Nrc1 contains no obvious functional domains (Figure 3A). To address this, we generated an Nrc1.TAP strain to isolate any associated proteins by sequential purification/LC-MS. This identified all three subunits of the CKII holoenzyme (Cka1, Ckb1, and Ckb2) and five TAFs (TBP-associated factors) as binding partners (Figure 4A; Table S1). The association with CKII was of particular interest because the human form of this kinase hyperphosphorylates four subunits of condensin I in interphase, inhibiting its supercoiling activity (Takemoto et al., 2006). This kinase-substrate relationship appears conserved across evolution, with proteomic analyses identifying phosphopeptides corresponding to consensus CKII substrates on the non-SMC subunits of budding yeast condensin (Bazile et al., 2010; Beltrao et al., 2009; Smolka et al., 2007). Fission yeast condensin contains > 100 consensus CKII sites (SXXE/D: high-threshold predictions by GPS 2.1 online; Xue et al., 2008), so we used a genetic approach to investigate any potential relationship between these factors. The CKII α-kinase subunit (Cka1) is essential for viability, but both β-regulatory subunits (Ckb1 and Ckb2) are individually redundant (Roussou and Draetta, 1994), and *ckb1*Δ or *ckb2*Δ each rescued condensin mutants (Figure 4B), with *ckb1*Δ also improving the mitotic chromosome architecture achieved by *cut3-477* alone (Figure S1K). This penetrant rescue of mutant condensin suggests a core role for the kinase, despite its multiple substrates (Filhol and Cochet, 2009). The relative strength of rescue (*ckb1*Δ > *ckb2*Δ) may reflect the differential contribution of each β-regulatory subunit to activity of the α-kinase in vivo (as suggested by cell growth, with *cka1*Δ lethal > *ckb1*Δ sick > *ckb2*Δ WT-like; Figure 4B).

CKII is a promiscuous enzyme, so in vivo specificity is achieved by associated proteins targeting the kinase to distinct subcellular locations (Filhol and Cochet, 2009). In this manner, Cka1 binding at the core centromere through mitosis resembled and was partially dependent on Nrc1 (Figures 3C and 4C). Therefore, we asked whether the NCT (Nrc1-CKII-TAFs) complex might mediate CKII-delivery, and thus whether the kinase is found in this context at specific genomic regions. To this end, we used ChIP sequencing (ChIP-seq) to analyze three representative complex subunits (Nrc1, Cka1, and Taf7) and identified > 2,000 peaks for each factor (Figures 4D, 4E, and S3–S5; Table S2), with quantitative PCR (qPCR) confirming the expected enrichment at ten test locations (Figures 4F and S4). Initial comparisons suggested a limited correlation between specific regions of Nrc1, Cka1, and Taf7 enrichment across the epigenetically complex centromere (Figures 4D and S3; see Discussion), but a striking correspondence between all three factors along the chromosome arms (e.g., Figures 4E and S3). On considering the major peaks of Nrc1, Cka1, and Taf7 (approximately top 15%: see Table S2), we noted that essentially all were located at transcription start sites (TSSs ± 250 bp). Furthermore, all three proteins were consistently coenriched at these locations, with ~52%, 54%, and 47% of their respective major peaks in the context of NCT (Figure 5A). This strong overlap was maintained when all peaks associated with a TSS were considered, with the percentage of each within NCT > 42% in the case of Nrc1 and Cka1 (Figure 5B). Permutation testing (randomly sampling 10^6 TSS sets of equal size to those bound by Nrc1, Cka1, and Taf7) estimated the chance overlap rate for their major peaks at 1.43 ± 1.19 (208 observed; $p < 10^{-6}$) and all peaks at 54.24 ± 6.96 (573 observed; $p < 10^{-6}$). This highly significant disparity between chance and observation supported a direct relationship between all three factors.

We considered that NCT could represent TFIID, a component of the RNA polymerase II (RNApII) preinitiation complex: Bdf1 (a potential homolog of Nrc1), CKII, and the TAF proteins all copurify in this context from budding yeast (Auty et al., 2004; Matangkasombut et al., 2000). However, multiple lines of evidence identify NCT as a distinct complex. For example, NCT is associated with 194 of the 215 genes encoding structural RNAs (i.e., 5S rRNA, snoRNA, snRNA, and tRNA: Figures 5C–5F), many of which are transcribed by RNApIII (Roberts et al., 2003). Furthermore Nrc1, Cka1, and Taf7 in comparison to TBP (and by extension, TFIID) are not restricted to promoters, and their major peaks cover a much greater area (~1.2 kb NCT versus ~450 bp TBP: Figure S5). Indeed, the peak structure of specific subunits may indicate how NCT could associate with specific regions. Of Nrc1, Cka1, and Taf7, the last most closely resembled TBP (e.g., Figure 4E). This was particularly obvious at the divergently transcribed histone loci, where TBP and Taf7 were most highly enriched over the central promoter region, with Nrc1 and Cka1 also abundant across both gene bodies (Figures 5G and S6). In contrast, Nrc1 and Cka1 spreading was unidirectional along the unpaired *hta2*$^+$ (Figure 5G). Thus, the TAF subunits (represented by Taf7) may mediate NCT recruitment to promoter-bound TBP, and the complex could then "spread" over a wider area via the Nrc1 bromodomains binding H4ac (and/or other acetyl marks), perhaps also promoted by active transcription. In this manner, Nrc1 would stabilize, rather than target, the association of NCT with chromatin, making best use of the low-affinity BD-Kac interaction (e.g., Figure 3F).

Condensin and the NCT Complex Bind Similar Genomic Locations but Not at the Same Time

As above, mutants in NCT rescue those in condensin (e.g., Figures 2D, 3D, and 4B). To further investigate if this indicated a direct regulatory relationship, we tested if the two complexes shared a preference for locations additional to the core centromere (e.g., Figures 2D, 3C, and 4C). Condensin is extrachromosomal for much of the cell cycle (Nakazawa et al., 2008), so we used cell-cycle synchronized ChIP-seq of identify sites of complex binding (represented by the Cut3 subunit) at various stages through mitosis. As expected, Cut3 was highly enriched at the core centromere in metaphase/anaphase and then rapidly dissociated (Figures 6A, 6B, and S3D). Cut3 associated with comparable kinetics at ~1,759 sites across the chromosome arms, including its previously reported preference for the structural RNAs (D'Ambrosio et al., 2008; Haeusler et al., 2008; Tanaka et al., 2012) (144 of the 215 genes; p value < 2.2×10^{-16};

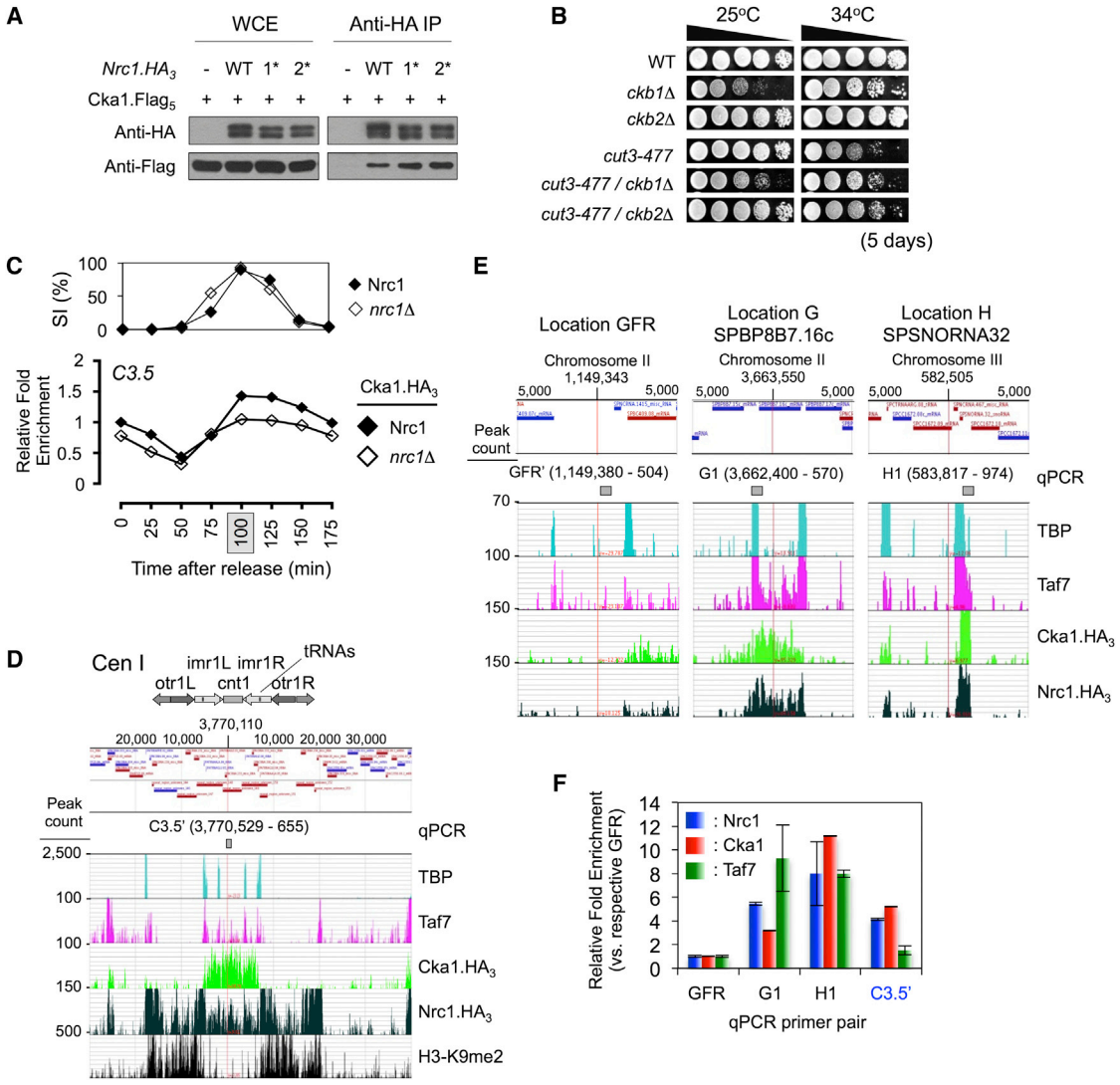

Figure 4. Identification and Initial Characterization of the NCT Complex

(A) Nrc1 (independent of its BDs) coprecipitates Cka1 (see also Table S1). Epitope tagged factors from WCEs or IPs were immunoblotted as indicated.

(B) Deletion of the CKII regulatory β subunits rescues *cut3-477* (*ckb1*Δ > *ckb2*Δ: see also Figure S1K). Strains were spotted as 10-fold serial dilutions onto YES plates.

(C) Cka1 occupancy at the core centromere through mitosis is partially dependent on (and parallels: see Figure 3D) Nrc1. All ChIP signals are expressed relative to WT T0 (set to 1).

(D) Nrc1, Cka1, and Taf7 enrichment across the chromosome 1 centromere (see also Figure S3). ChIP-seq data from asynchronous cells were loaded into GenPlay as individual tracks relative to the annotated *Sp* genome (for dynamic visualizations of each track of the GenPlay project, see Data S1). TBP identifies the tRNAs that demark the inner/outer centromere and pericentromeric boundary; H3-K9me2 identifies the heterochromatic outer repeats. Upper schematic depicts CEN1 structure; lower track genomic elements (e.g., genes or tRNAs) in the ~70 kb window region.

(E) Nrc1, Cka1, and Taf7 colocalization at representative locations on the chromosome arms (see also Figure S3). Each 10 kb window is named for the centered genomic feature. *GFR* encompasses the condensin-free region used as a background control for ChIP (e.g., Figure 2C).

(F) ChIP confirms ChIP-seq predictions of Nrc1, Cka1 and Taf7 enrichment at the chromosome arms (*GFR'*, *G1*, and *H1*) and core centromere (*c3.5'*). Samples were prepared from asynchronous cells and analyzed by qPCR (rather than duplex PCR as done previously) with primers flanking the regions indicated in (D) and (E). The enrichment of each factor at each location is normalized to *GFR* (set to 1 for each IP).

Figures 6C, S3H, and S7; Table S2). Of note the specific areas of Cut3 enrichment across the chromosome arms were strongly reminiscent of those bound by NCT (e.g., Figures S3 and S6). As an example, Cut3, Nrc1, Cka1, and Taf7 share a preference for 425 TSS-containing regions (Figure 6D); >60-fold more than expected by chance (permutation testing 10^6 events: 6.49 ± 2.52). Furthermore, the overlap between [Cut3: NCT] at these locations encompassed > 80% of [Cut3: Cka1 alone] (Figure 6E), suggesting the central importance of NCT to any relationship between CKII and condensin.

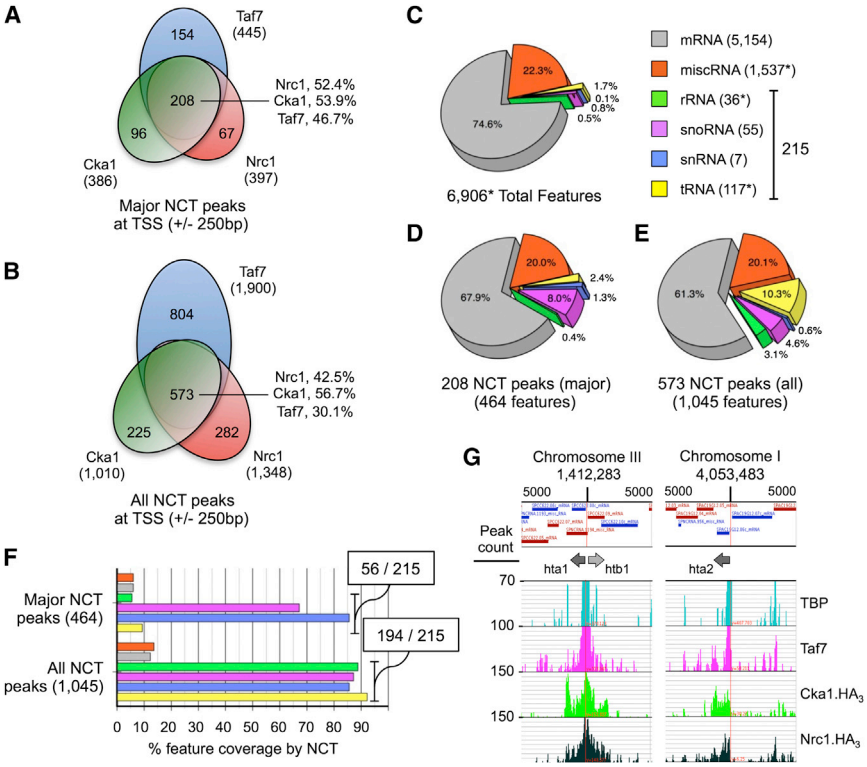

Figure 5. NCT Complex Is Enriched at the Structural RNA Genes

(A and B) Nrc1, Cka1, and Taf7 are coenriched at transcription start sites (TSSs ± 250 bp). Peaks were identified, localized, and quantified by optimized parameters (see Table S2).

(C) 6,906 transcribed RNA species are annotated to the fission yeast chromosome arms. These are classed by type and presented by percentage.

(D–F) The structural RNAs are overrepresented as NCT peaks. Data are presented as the percentage of NCT peaks (major/all) per feature, and percentage coverage of each feature (specific NCT peaks were assigned to all overlapping features when required). For (F), the Fisher's exact test (by *R*) was used to estimate the probability of the observed enrichment at structural RNAs: major NCT peaks, p value 5.5×10^{-16}; all NCT peaks, p value $< 2.2 \times 10^{-16}$ (the smallest value *R* will provide).

(G) Nrc1, Cka1, and Taf7 enrichment across the histone genes (see also Figure S6). Each 10 kb test window is named for the centered histone locus. In each case, TBP and Taf7 are most enriched at the intergenic promoter region, whereas Nrc1 and Cka1 bind across the gene (note the unidirectional spread at unpaired *hta2*$^+$).

Finally, we asked if condensin and NCT co-occupy their preferred locations along the chromosome arms through mitosis. To this end, we subjected Nrc1 to synchronous ChIP-seq and compared its enrichment to that of Cut3 at each cell-cycle stage. As expected from direct ChIP analyses (e.g., Figures 2D and 6A), Nrc1 levels across the centromere were lowest at metaphase (T_{75}) when Cut3 binding was at its peak (Figures 6B, 6B′, and S3). However, a similar pattern was observed across the chromosome arms, with Nrc1 delocalizing from its preferred sites through mitosis and returning in G_1/S (Figures 6C′ and S3). This profile extended through the NCT complex, with Nrc1, Cka1, and Taf7 occupancy at representative sites increasing as cells pass through mitosis (Figures 6F and S4). We note that such a pattern would colocalize CKII with its potential condensin substrate as cells complete mitosis and chromosome decondensation occurs (Figure 7).

DISCUSSION

In this study, we uncover and characterize a potential mechanism for the regulation of genome condensation at mitosis. We identify the Hat1-Mis16 acetyltransferase complex, show that this contributes to the global acetylation of histones H3 and H4, and establish its role in the mitotic regulation of these marks at the centromere (Figure 2). We demonstrate the selectivity of Nrc1-BD1 for H4ac and show that Nrc1 requires its bromodomains for efficient recruitment to the centromere (Figure 3). We then characterize the NCT complex (Figures 4 and 5) and present several lines of evidence that Hat1-Mis16 and NCT regulate the functionality of mitotic chromosomes via the condensin complex. This includes the ability of mutants in *hat1*, *mis16*, *nrc1*, and *CKII* to restore segregation-competent chromosomes when condensin is defective (e.g., Figures 1, 2G, 3D, 4B, S1A, and S1K), and the extensive colocalization of NCT with condensin (e.g., Figures 6, S3, and S6). However, the latter is not constitutive: NCT and condensin primarily overlap in early and late mitosis, and it is tempting to speculate that this temporally limited colocalization is directly regulatory to genome condensation at these cell-cycle stages (Figure 7).

The Regulation and Role(s) of NCT Complex

Our identification of the NCT complex is supported by three independent approaches: copurification, shared mutant phenotypes, and extensive subunit colocalization (e.g., Figures 4 and 5). Given its tandem bromodomains, it is likely that the Nrc1 subunit contributes to NCT targeting and/or binding at specific genomic locations (Filippakopoulos et al., 2012; Wang et al., 2013). In this regard, we show that both Nrc1 BDs are functional (Figure 3D) and required for efficient Nrc1 recruitment to the centromere (Figure 3C). Furthermore, BD1 shows selective binding to the hyperacetylated H4 N terminus (Figures 3E, 3F, and S2E–S2G), and Nrc1 levels track centromeric H4ac levels through mitosis (Figure 2D). However, the low affinity of this interaction (Figure 3F) suggests that additional, as yet unidentified, entities (e.g., linked to active transcription; Figure 5G) contribute to the efficient in vivo association of NCT with chromatin. These could be recognized by other members of the NCT complex at specific genomic locations and/or cell-cycle stages.

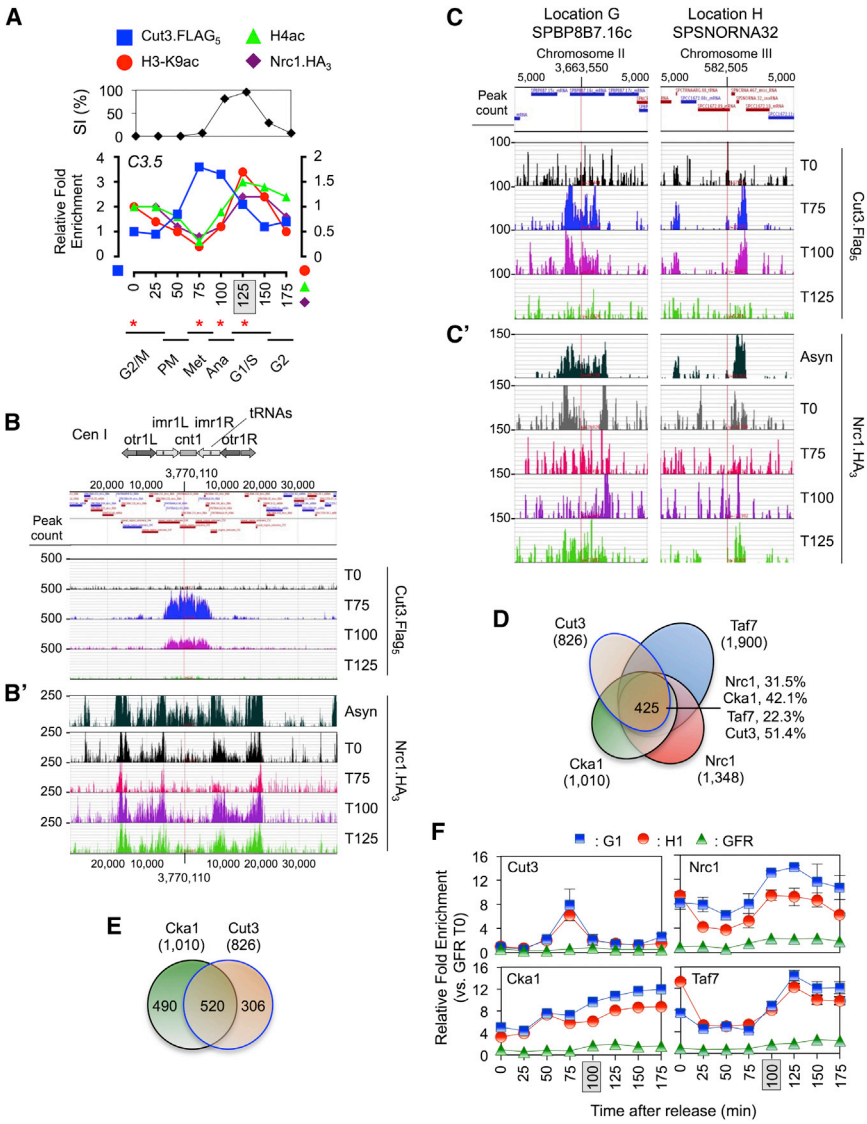

Figure 6. Condensin and the NCT Complex Bind Similar Locations but Are Temporally Anticorrelated through Mitosis

(A) Cells were synchronized (peak septation shaded) and ChIPed (as in Figure 2C), and samples from the indicated time points (*) were used to prepare ChIP-seq libraries. Cell-cycle stages are predictions. PM, prometaphase.

(B and B′) Cut3 and Nrc1 binding across the chromosome 1 centromere are mitotically regulated (see also Figure S3): Cut3 levels are highest at metaphase (T_{75}) when Nrc1 levels are at their lowest. ChIP-seq data from synchronized populations (time points as in A) were loaded into GenPlay as individual tracks relative to the annotated *Sp* genome (Data S1). Asyn, asynchronous.

(C and C′) Cut3 and Nrc1 binding at similar locations across the chromosome arms are mitotically regulated (see also Figure S3). Note the different y axis scales in (B) and (C).

(D and E) The NCT (asynchronous; TSS ± 250 bp) and condensin (metaphase; TSS ± 500 bp) occupy similar locations. Cka1 is found in the context of NCT at >80% of these [Cka1$^+$/condensin$^+$] sites (425/520).

(F) Cut3, Nrc1, Taf7, and Cka1 binding are mitotically regulated (determined by qPCR with the primers in Figures 4D and 4E). Samples were prepared from synchronized populations, and all data (mean ± SD) were normalized relative to the respective *GFR*-T_0 (set to 1).

NCT as a Regulator of Condensin Function

We have largely focused on the potential role of NCT in mitotic genome function and considered a range of possible mechanisms by which the transient overlap between NCT and condensin in early and late mitosis could directly regulate chromosome condensation/decondensation. Recent studies show that human Brd4-isoform B mediates the recruitment of condensin II during DNA damage (Floyd et al., 2013). This is of particular note because Brd4 is the closest human homolog of Nrc1: both contain tandem bromodomains share a specificity for H4ac and are selectively inhibited by JQ1 (Filippakopoulos et al., 2012; Filippakopoulos et al., 2010; data not shown). However, Nrc1 does not contain the responsible C-terminal condensin II-interaction domain in Brd4-isoform B, *Sp* only contains the condensin I complex, and the suppressive genetic interactions of *nrc1* and *condensin* mutants do not support a positive role for Nrc1 in condensin recruitment. In an alternate interpretation, their genetic interactions and mitotically regulated binding profiles could indicate that NCT and condensin compete for occupancy at specific locations, such that NCT must be removed at prometaphase to allow condensin to load, and its return after anaphase displaces condensin. However, evidence argues against such a relationship: NCT and condensin bind related rather than identical sites (e.g., compare the peaks in Figures

This study does not specifically address any role(s) for NCT in interphase cells, although its specific sites of enrichment are highly suggestive of function. For example, the almost total coverage of structural RNAs (194 of the 215 genes; Figures 5C–5F) indicates the importance of NCT to these locations, where the concomitant recruitment of CKII may contribute to the positive regulation of RNApIII transcription (Ghavidel and Schultz, 2001). It also remains to be determined if NCT functions exclusively as a CKII-delivery platform, although it is almost certainly not the only means to recruit this promiscuous kinase to chromatin. CKII is targeted to distinct subcellular locations by a range of associated proteins (Filhol and Cochet, 2009), and NCT likely differentially contributes to the total kinase pool at various chromosomal locations: this would help to explain the peak patterns of Cka1 versus Nrc1 and Taf7 at distinct regions, including the core centromere (Figures 4D and S3), and the only partial dependence of Cka1 on Nrc1 at this location (Figure 4C).

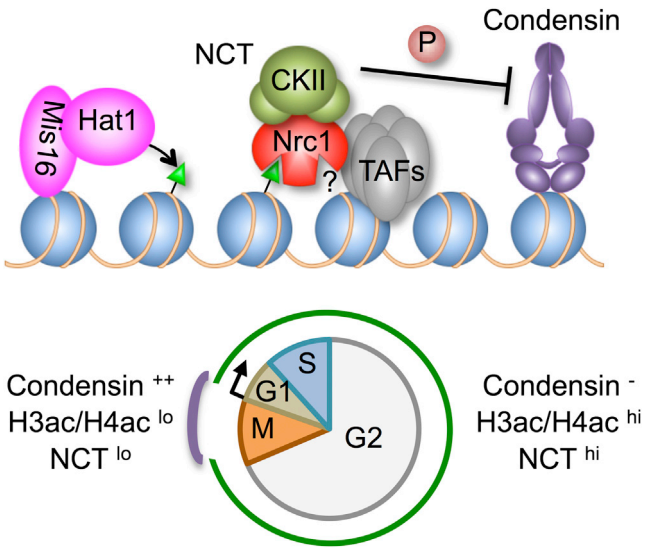

Figure 7. NCT as a Condensin Regulator

H3ac/H4ac at the core centromere are catalyzed by the Hat1-Mis16 acetyltransferase. H4ac is recognized by Nrc1-BD1 to stabilize the association of NCT with chromatin. CKII phosphorylates condensin to inhibit its supercoiling activity (Takemoto et al., 2006). Though NCT and condensin share a preference for many chromosomal locations, their binding is regulated to anticorrelate through mitosis: this gives potential control over genome condensation and decondensation.

S3 and S6), and Cut3 recruitment is comparable in WT and *nrc1* cells (e.g., Figure 3C).

We rather favor a mechanism that accommodates all our findings and builds on the observation that human condensin I is phosphorylated, and thus catalytically inhibited, by CKII (Takemoto et al., 2006). In this manner, any colocalization of kinase and substrate would be directly regulatory, such that the dissociation of NCT at early mitosis would relieve any CKII-mediated inhibition of condensin to control chromosome condensation, whereas its return in G_1 would contribute to decondensation (Figure 7). This model would also accommodate the extensive literature relating mitotic kinase activity to condensin function (reviewed in Bazile et al., 2010).

Human condensin I is constitutively hyperphosphorylated, though the specific sites and responsible enzymes differ at interphase and mitosis. Phosphorylation at interphase (when condensin is primarily cytoplasmic) is mediated by CKII, whereas that at mitosis (after transport into the nucleus) is mediated by the mitotic kinases Cdk1 (Cdc2), Aurora B (Ipl1), and Polo (Cdc5) (Bazile et al., 2010; St-Pierre et al., 2009; Takemoto et al., 2006, 2007). The primary role of phosphorylation may be to control the catalytic activity of condensin, whose intrinsic ability to supercoil DNA is inhibited by CKII but activated by the mitotic kinases (Bazile et al., 2010). Of particular importance, the CKII-dependent inhibition of condensin is dominant over its Cdk1-dependent activation (Takemoto et al., 2006). This could explain how budding yeast condensin can remain constitutively associated with chromosomes (D'Ambrosio et al., 2008; Wang et al., 2005): their condensation at mitosis is not the direct consequence of condensin binding but rather occurs after appropriate activation.

Chromosome condensation initiates in early mitosis, suggesting that condensin is fully activated in response to low CDK1 levels (Bazile et al., 2010). Spurious activation is a possible consequence of such ultrasensitivity, so condensin arrives from the cytoplasm painted with CKII-mediated inhibitory phosphorylations (Bazile et al., 2010; Takemoto et al., 2006). However we now show that CKII (both within and independent of the NCT) also occupies many condensin binding sites at the core centromere and chromosome arms (e.g., Figures 6C–6E and S3). Any colocalization of inhibitory CKII with condensin at early mitosis would allow NCT to regulate the initiation of condensation and could explain the increased rate of defective anaphases in *nrc1*Δ and *ckb1*Δ cells (Figures 1D and S1K).

As mitosis proceeds, full condensin activation/chromosome condensation would require NCT to dissociate from chromatin, and any inhibitory CKII-mediated phosphorylations to be removed (possibly by PP2A or PP1 phosphatases; Vagnarelli et al., 2006; Xing et al., 2008). The precise means by which NCT is displaced prior to metaphase is unknown, although it may be a combination of mitotic phosphorylation (which drives the global displacement/relocalization of chromatin associated factors [Zaidi et al., 2010] and reduced acetylation [Kruhlak et al., 2001; Sasaki et al., 2009]) (Figures 2D and S1I). Of note, NCT does not entirely dissociate from chromatin, and indeed Cka1 levels on the chromosome arms gradually climb through mitosis from a nadir at G_2/M (Figure 6F). However, CKII is also phosphorylated, and inhibited, by CDK1 (Litchfield et al., 1992; St-Denis et al., 2009), so any colocalization of CKII with condensin through metaphase may have limited impact.

The means by which chromosome decondensation occurs at mitotic exit is poorly understood, although the kinetics of NCT return to condensin enrichment sites (Figure 6F) could suggest its role in this process. The return of NCT likely results from a combination of reduced mitotic kinase levels (Zaidi et al., 2010), increased histone acetylation (Kruhlak et al., 2001; Sasaki et al., 2009) (Figures 2D and S1), and the increased availability of Nrc1 (whose transcription peaks in G_1; Peng et al., 2005). This would localize CKII with condensin after chromosome separation, allowing the kinase to inactivate the ATPase complex and directly promote decondensation. Studies to address each of these predictions are currently under way but will require the development of approaches to monitor the condensation of specific locations at various mitotic stages.

EXPERIMENTAL PROCEDURES

Materials

Antibodies, yeast strain genotypes, and ChIP oligonucleotides are in Table S3.

Bromodomain Binding to Histone Peptide Arrays

To produce recombinant forms of Nrc1, a region encompassing both bromodomains (residues S199–G526) was C-terminally fused to GST in pGEX-4T, heterologously expressed in *E. coli*, and purified by glutathione-Sepharose affinity chromatography (Fuchs et al., 2011). Recombinant proteins (WT; BD1* [$PDYP_{266-269}AAAA$]; BD2* [$PDYF_{428-431}AAAA$]; or BD1*/BD2*) were used to probe a histone-tail peptide array with combinations of posttranslational modifications and areas of enrichment identified from heatmaps of the

normalized mean intensity. All data were generated from a minimum of two arrays with 12–24 individual spots per peptide (Figures 3E and S2E–S2G). Array preparation, binding conditions, and data analysis were as previously described (Fuchs et al., 2011; Rothbart et al., 2012).

Cell-Cycle Synchronization

Cell-cycle synchronization, ChIP, and septation analyses were performed after arrest by *cdc25-22* (Kim et al., 2009) (Figure S1B).

ChIP-Seq

Input or ChIP samples were converted to bar-coded libraries by adaptor addition (Quail et al., 2008). In brief, each sample was quantified, end-repaired (End-It kit, Epicenter Biotechnologies), an A-overhang added, and ligated to one of a set of 12 barcode-specific adaptor primers. The resulting libraries were size selected (600 ± 50 bp), amplified by 18 cycles of PCR, purified with SPRI beads (Agencourt AMPure XP; Fisher Scientific), quantified on a high-sensitivity DNA chip by 2100 bio-analyzer (Agilent Technologies), mixed at equivalent concentrations, and multiplexed at 12 samples per Illumina HiSeq 2000 lane.

Data images from 100 bp runs (single or paired-end) were processed by the Illumina Sequence Control and Pipeline packages. ChIP-seq data were loaded into GenPlay (v533) as individual tracks (50 bp windows; normalized and INPUT subtracted; for a dynamic visualization of the discussed tracks, see Data S1) relative to the annotated fission yeast genome (Sanger Center release pombe090511) (Lajugie and Bouhassira, 2011; Zang et al., 2009). Peaks for comparison and assignment to genomic features were automatically identified/quantified by optimized parameters (see Table S2). The location, level, and timing of each peak, as well as their association with different features, were described by summary statistics and standard modeling approaches (using Microsoft Access and R). Permutation testing was used to measure the chance overlap rate between specific sets of ChIP-seq peaks by randomly sampling 10^6 sets of equal size to those under test.

In Situ Gene Tagging and Mutagenesis

Constructs for epitope tagging were assembled by PCR megapriming from *Sp* genomic DNA or plasmid templates (Janke et al., 2004; Keogh et al., 2002), with products transformed/targeted by homologous recombination in the appropriate fission yeast backgrounds. Mutations at the Nrc1 bromodomains (BD1*, $PDYP_{266\text{-}269}AAAA$; BD2*, $PDYF_{428\text{-}431}AAAA$) were created by two-step marker replacement (Mehta et al., 2010). Nrc1 levels were modulated by "knocking in" alternate promoters of predicted expression outputs: $fba1_{pr}$ > $lys4_{pr}$ > $ade4_{pr}$ (Hiraoka et al., 2009), and the resulting expression was determined by immunoblotting (Figure S2A).

Isothermal Titration Calorimetry

ITC measurements were conducted at 25°C with a MicroCal Auto-ITC200 in 1 × PBS (pH 7.6) (2.7 mM KCl, 1.5 mM KH_2PO_4, 140 mM NaCl, 8 mM Na_2HPO_4). Recombinant forms of the Nrc1 bromodomains (residues S199-G526; see Figure 3A) were dialyzed in 1 × PBS (pH 7.6) and brought to 30 μM. Peptides were lyophilized and resuspended in 1 × PBS (pH 7.6) at 400 μM. Titrations were performed with 20 × 2 μl peptide injections at 180 s intervals using a reference power of 8 μcal/s. Results were analyzed using the MicroCal Origin software, and the Isotherm (μcal/s) and integrated data (Kcal/Mole of injectant) for each titration were presented (Figure 3F).

Microscopic Analysis of Mitotic Chromosomes

Cells were grown in rich media and briefly (<30 s) fixed in 100% methanol at −80°C. DNA and microtubules were visualized by DAPI staining and antitubulin immunoblotting. Anaphases were scored as defective if chromatin was observed near the metaphase plate after the bulk of separation had occurred (Kim et al., 2009). Data were presented as the percentage (mean ± SD) of late anaphase cells with chromosome segregation defects (counted >100 cells per population; e.g., Figure 1D).

PEM-2 Screening to Identify Second-Site Suppressors of *condensin* Mutants

The *PEM-2* approach (Roguev et al., 2008) was used to place *ts* alleles of each condensin ATPase subunit (*cut3-477* [S1146P] or *cut14-208* [S861P]; Sutani and Yanagida, 1997) in the context of 3,002 fission yeast gene deletions (~75% of the nonessential *Sp* genome). Viable double mutants were isolated at a permissive temperature for each *condensin* allele (25°C), and suppressors were identified by replica plating and improved growth at restrictive temperatures (34°C and 37°C, e.g., Figure 1B). To identify deletions that might suppress *condensin* by a nonspecific mechanism, parallel screening was performed with *cdc25-22*. Specific suppressors of interest were confirmed by direct crossing to a range of *condensin* alleles (*cut3-477*, *cut14-208*, and *cnd2-1* [A114T]), tetrad dissection, and spot testing (e.g., Figure 1C) prior to further investigation.

Protein Complex Purification/Identification of by LC-MS

C-terminally TAP-tagged proteins ($yfg1^+$-CBP-TEV-2xProtein A) were purified and associated proteins identified by LC-MS as previously (Kim et al., 2009; Silva et al., 2012).

Protein Immunoprecipitations

Cultures were grown to an OD_{600nm} ~1.0 and pelleted for the preparation of whole-cell extracts (WCEs). Cells were disrupted with glass beads in prechilled (4°C) lysis buffer (20 mM HEPES [pH 7.6], 200 mM KOAc, 10% Glycerol, 1 mM EDTA) supplemented with protease inhibitors (1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin-A). WCE samples were quantified, standardized for concentration, and $nrc1.HA_3$ or Hat1.$FLAG_5$ immunoprecipitated with anti-HA (12CA5) or anti-FLAG (FLAG-M2; Sigma-Aldrich) as previously described (Keogh et al., 2002).

qPCR

ChIP samples were prepared as above and qPCR data obtained with an iCycler (Bio-Rad), SYBR green (Molecular Probes), Platinum Taq (Invitrogen), and the primer sets in Table S3. Quantitations were as previously described (Silva et al., 2012), with all enrichments expressed as a percentage of input or relative to a gene-free region (GFR: Chromosome II 1,149,380–1,149,504) that shows no specific enrichment of TBP, Nrc1, Cka1, Taf7, or Cut3 in ChIP-seq (Figure S3).

Whole-Cell Extracts for Immunoblotting

WCEs were isolated with trichloroacetic acid (Mehta et al., 2010), and supernatants were analyzed by immunoblotting.

ACCESSION NUMBERS

All sample data have been deposited in the NCBI Gene Expression Omnibus (Edgar et al., 2002) and are accessible under accession number GSE53955.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, three tables, and one data set and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.01.029.

AUTHOR CONTRIBUTIONS

R.M. and S.B.R. contributed equally to this work. H.-S.K., R.M., S.B.R., V.V., T.K., N.J.K., J.S.F., B.D.S., W.E., and M.-C.K. conceived the experiments. H.-S.K., R.M., S.B.R., A.C.S., V.V., E.R., T.K., A.R., C.J.R., J.X., H.J., and J.S.F. performed the experiments. H.-S.K., R.M., S.B.R., A.C.S., V.V., T.K., C.J.R., K.G.H., J.F.G., N.J.K., B.D.S., E.E.B., W.E., and M.-C.K. analyzed the data. H.-S.K., R.M., S.B.R., and M.-C.K. wrote the manuscript.

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